RESEARCH LETTER

Isolation and identification of cobalt- and caesium-resistant bacteria from a nuclear fuel storage pond

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Abstract

One of the issues facing the nuclear power industry is how to store spent nuclear fuel which is contaminated with radionuclides produced during nuclear fission, including caesium ($^{134}Cs^+$, $^{135}Cs^+$ and $^{137}Cs^+$) and cobalt ($^{60}Co^{2+}$). In this study, we have isolated Co^{2+}- and Cs^+-resistant bacteria from water collected from a nuclear fuel storage pond. The most resistant Cs^+ and Co^{2+} isolates grew in the presence of 500 mM CsCl and 3 mM $CoCl_2$. Strain Cs67-2 is resistant to fourfold more Cs^+ than *Cupriavidus metallidurans* str. CH34 making it the most Cs^+-resistant strain identified to date. The Cs^+-resistant isolates were closely related to bacteria in the *Serratia* and *Yersinia* genera, while the Co^{2+}-resistant isolates were closely related to the *Curvibacter* and *Tardiphaga* genera. These new isolates could be used for bioremediation.

Introduction

Nuclear power is a major contributor to electrical energy production in many countries; however, it produces a significant amount of toxic environmental waste. The radionuclide $^{60}Co^{2+}$ has a half-life of 5.3 years and is produced during the nuclear fission process by thermal neutron bombardment of the natural isotope, which is present in a number of steel containing components of nuclear reactors. Radioactive Cs^+ is a fission product and its isotopes $^{134}Cs^+$, $^{135}Cs^+$ and $^{137}Cs^+$ have half-lives of 2.1 years, 2.3 million years and 30 years, respectively (Kobayashi & Shimizu, 1999). One of the major problems at nuclear power plants is the disposal of spent nuclear fuel that is no longer effective for producing a nuclear reaction and hence needs to be safely disposed. Spent nuclear fuel must be kept in underwater racks to cool prior to final storage. Storage ponds use deionized water to cool the spent fuel and protect against radiation.

The main health concern associated with these radionuclides is the increased risk of cancer due to the effects of beta and gamma radiation. In addition to radiation, the toxicity of Co^{2+} and Cs^+ is also detrimental to human health. Cs^+ enters the body through ingestion, and due to its physiochemical resemblance to K^+, it is transported around the body via K^+ transport systems (Kuwahara *et al.*, 2011) interfering with K^+ homeostasis. It has been proposed that the mode of toxicity of Cs^+ is by the depletion of K^+ (Avery, 1995). Co^{2+} also enters the body via ingestion, where it competes with Fe during synthesis of Fe-S clusters in essential metabolic proteins, resulting in their inactivation (Ranquet *et al.*, 2007; Barras & Fontecave, 2011). Co^{2+} toxicity can cause various health problems such as contact dermatitis, pneumonia, allergic asthma and lung cancer (Barceloux, 1999), while Cs^+ toxicity is associated with fatigue, muscle weakness, palpitations and arrhythmia (Melnikov & Zanoni, 2013). The potential negative health effects associated with Co^{2+} and Cs^+ from spent nuclear fuel necessitate the requirement for removal strategies; bacteria that can survive in environments with high concentrations of Co^{2+} or Cs^+ radionuclides could be useful for nuclear fuel remediation.

Materials and methods

Sample site

A water sample from an external storage pond at Sellafield Ltd (Cumbria, UK) was obtained from 5 m below the surface to enrich and isolate bacteria resistant to Co^{2+} and Cs^+.

Isolation of Co- and Cs-tolerant microorganisms from enrichment cultures

Duplicate enrichment cultures were set up in 10 mL of R2A medium (Reasoner & Geldreich, 1985) where either $CoCl_2$ was added to a final concentration of 0.5, 0.75, 1 or 2 mM, or CsCl was added to a final concentration of 25, 50, 75 or 100 mM. *Escherichia coli* str. K38 is considered to be neither metal resistant nor sensitive and has a minimum inhibitory concentration (MIC) of 1 mM for $CoCl_2$ and > 50 mM for CsCl (Nies, 1999); therefore, representative concentrations were used for the enrichments. One milliliter of the pond water sample was added to each tube which was incubated at either 10 or 28 °C. As the storage pond is outside, the temperature is not regulated and the water temperature is affected by the weather. The water temperature at the time of sampling was 21.2 °C; enrichments were conducted at 10 and 28 °C to account for seasonal changes in temperature associated with the UK climate and the heating of the pool caused by the spent fuel. Following incubation, a 1% inoculum from the enrichment culture containing 1 mM $CoCl_2$ or 100 mM CsCl in which growth was observed (by turbidity) was transferred to fresh broth containing the same and a twofold higher concentration of $CoCl_2$ or CsCl and incubated at the same temperature. The subsequent enrichments were plated onto R2A agar containing the same concentration of $CoCl_2$ or CsCl as the original culture. Colonies of unique morphology were picked and streaked onto fresh R2A agar containing $CoCl_2$ or CsCl. This process was repeated twice more to ensure pure cultures were obtained.

Restriction fragment length polymorphism (RFLP) analyses

PCR was undertaken using the universal primers 63f and 1387r (Lane, 1991) to amplify the 16S rRNA gene of the isolates. PCR products were microdialysed against MQ water for 45 min using a MF™-Millipore membrane filter with a pore size of 0.025 μm to remove salts from the solution. Each isolate was digested with the 4-bp cutter restriction enzymes HhaI, MspI and RsaI (Promega) following the manufacturer's guidelines. Following digestion, PCR products (10 μL) were visualized by electrophoresis on 2% agarose gels and the restriction profiles analysed.

16S rRNA gene sequencing

DNA sequencing was performed by Source Bioscience using an ABI 3730xl 96 capillary Genome Analyser analysis system. The template of each isolate was provided as a purified PCR product at a concentration of 15 ng μL^{-1} and in a volume of 5 μL. For all isolates, the primers 27f and 1492r (Lane, 1991) were used for PCR amplification and sequencing.

Sequence alignment and phylogenetic analysis

Nucleotide sequences were trimmed and aligned using MUSCLE (Edgar, 2004) using default settings. BLAST searches of the 16S rRNA gene sequence against the 16S ribosomal RNA sequences (*Bacteria* and *Archaea*) database were used to determine which bacteria the isolates were closely related to. Using the CLASSIFIER tool of the Ribosomal Database Project (Wang *et al.*, 2007) and the EZTAXON-E Database (Kim *et al.*, 2012), isolates were identified to the family or genus level. Phylogenetic analysis and trees were constructed with MEGA 5.05 (Tamura *et al.*, 2011). Phylogenetic trees were constructed using the kimura-2-parameter algorithm and neighbour-joining method (Saitou & Nei 1987). Bootstrap values were from 100 resamples.

MIC of CsCl and $CoCl_2$ for water sample isolates

Cultures of the Co- and Cs-resistant bacterial isolates were grown in 10 mL of R2A medium and incubated at 28 °C until turbid. A dilution of the culture (25 μL) was spread plated onto half an R2A agar plate or R2A agar plates supplemented with either 100, 200, 300, 400, 500, 1000 mM CsCl, 0.5, 1, 2, 3, 4, 5 mM $CoCl_2$ or $NiCl_2$ or $ZnCl_2$; or 0.25, 1, 2, 3, 4 mM $CdCl_2$ or $CuCl_2$. The plates were incubated at 28 °C until colonies were visible. The effect of osmotic stress on the Cs-resistant isolates was tested in 10 mL R2A medium containing 300, 400, 500 and 700 mM NaCl.

Results and discussion

Eight Co^{2+}-resistant and four Cs^+-resistant isolates were purified from R2A agar containing 2 mM Co^{2+} or 100 mM Cs^+, respectively. One isolate (Cs60-2) was isolated from 10 °C, while the remainder were isolated from 28 °C enrichments. Three different RFLP profiles were observed with the Co^{2+} water sample isolates, and two different RFLP profiles were seen with the Cs^+ water sample isolates. Representatives of each phylotype were identified by sequencing a 1465-bp region of the 16S rRNA gene. The 16S rRNA gene sequences were used to construct a phylogenetic tree (Fig. 1) and submitted to the EZTAXON-E Database (Kim *et al.*, 2012) for taxonomic identification. All isolates were members of the *Proteobacteria*, with the Cs^+-resistant isolates belonging to the *Gammaproteobacteria*, whereas the Co^{2+}-resistant isolates were members of the *Alphaproteobacteria* and *Betaproteobacteria* (Fig. 1).

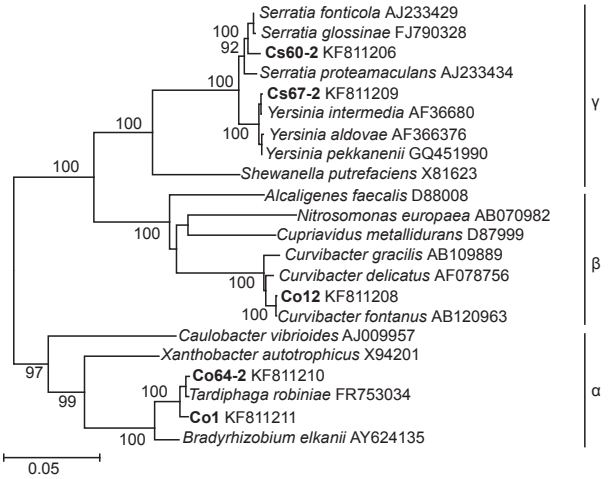

Fig. 1. Phylogenetic tree of the 16S rRNA genes from Co^{2+}- and Cs^{+}-resistant isolates and their phylogenetic relatives. Bootstrap values (per 100 trials) are shown. α – *Alphaproteobacteria*; β – *Betaproteobacteria*; γ – *Gammaproteobacteria*. Sequences were aligned with MUSCLE (Edgar, 2004) and the tree constructed using the kimura-2-parameter algorithm and neighbour-joining method with MEGA 5.05 (Tamura *et al.*, 2011). The tree was rooted with the 16S rRNA gene sequence of *Aeropyrum pernix* (not shown). Bar represents 0.05 substitutions per nucleotide position. GenBank accession numbers are shown.

The Co^{2+} isolates Co1 and Co64-2 are both closely related to *Tardiphaga robiniae*, while Co12 is closely related to members of the *Curvibacter* genera (Fig. 1). Cs^{+} isolates Cs60-2 and Cs67-2 are closely related to members of the *Serratia* and *Yersinia* genera, respectively (Fig. 1). Strains have been sent to the DSMZ for deposition.

The MIC for $CoCl_2$ and CsCl of the Co^{2+} and the Cs^{+} isolates was determined. The Co^{2+}-resistant isolates were all resistant to 2 mM $CoCl_2$, and one isolate (Co64-2) was able to grow in the presence of 3 mM $CoCl_2$. One of the Cs^{+}-resistant isolates (Cs60-2) grew in the presence of 0.5 mM $CoCl_2$; however, Cs67-2 was unable to grow in the presence of 0.5 mM $CoCl_2$. The Co concentration in the external storage pond was not measured. The Cs^{+}-resistant isolates grew in the presence of 300 mM (Cs60-2) and 500 mM (Cs67-2) CsCl; there are no known organisms able to tolerate these concentrations. Both Cs67-2 and Cs60-2 grew in the presence of 700 mM and 500 mM NaCl, respectively, indicating that Cs^{+} toxicity was not due to osmotic stress. Co^{2+} resistance is generally associated with Ni^{2+} and/or Zn^{2+} resistance via an efflux pump mechanism and can be either chromosomally or plasmid-encoded (Nies, 2003; Rodrigue *et al.*, 2005), while the mechanism of resistance to Cs^{+} is currently unknown. Apart from *Serratia*, which is known to be resistant to Cs^{+} (Paterson-Beedle *et al.*, 2006), none of the closest relatives of the identified isolates have been shown to be resistant to either Cs^{+} or Co^{2+}.

All of the other identified isolates in this work are related to genera that have been commonly isolated from water samples. The highly metal-resistant bacterium *Cupriavidus metallidurans* str. CH34 has a MIC of 25 mM for $CoCl_2$ and 125 mM for CsCl (Monsieurs *et al.*, 2011). The genome of *C. metallidurans* str. CH34 contains two chromosomes and two megaplasmids that contain a large number of genes implicated in the resistance to heavy metals (Mergeay *et al.*, 2003). It has been shown that genes on the megaplasmids can be activated by more than one metal; metal response genes are found on both megaplasmids pMOL28 and pMOL30 for Cd^{2+}, Ni^{2+}, Cu^{2+}, Pb^{2+}, Zn^{2+} and Co^{2+} (Monsieurs *et al.*, 2011). *Cupriavidus metallidurans* str. CH34 contains two clusters of heavy metal-resistance genes, *czc* located on pMOL30 (Liesegang *et al.*, 1993) and *cnr* located on pMOL28, that have been shown to be involved in Co^{2+} resistance which may explain its elevated MIC (Mergeay *et al.*, 1985; Nies *et al.*, 1987). In *E. coli*, Co^{2+} is transported into the cell by constitutively expressed divalent cation uptake systems of broad specificity, for example Mg^{2+} and Zn^{2+} transport systems (Nies, 1992); the *rcnA* gene encodes a membrane-bound protein that confers Ni^{2+} and Co^{2+} resistance and acts as an efflux pump to export the metals (Rodrigue *et al.*, 2005). Given the MIC to Co^{2+} of the isolates in this study (Table 1), it is possible that the mechanism for resistance for isolate Co12 is similar to that of *E. coli* as it cannot grow in the presence of Zn^{2+}. Although Cs^{+} is considered to be relatively nontoxic to microorganisms (Avery, 1995), isolate Cs67-2 grew in a medium with fourfold more CsCl than *C. metallidurans* str. CH34, identifying it as the most Cs^{+}-resistant bacterial strain known to date.

With the renewed interest in the nuclear fuel industry, there is also the need to develop technologies for the remediation of nuclear waste and contaminated materials. The nuclear industry needs to resolve the problem of long-term containment of radionuclide wastes and the

Table 1. MIC of metals for growth of *Cupriavidus metallidurans*, the Co^{2+}- and Cs^{+}-resistant isolates

	MIC (mM)					
Isolate	$CoCl_2$	$CsCl_2$	$NiCl_2$	$ZnCl_2$	$CuCl_2$	$CdCl_2$
C. metallidurans	25*	125*	13†	12†	3†	4†
Cs60-2	1	400	1	5	1	1
Cs67-2	0.5	1000	2	0.5	1	0.5
Co1	3	100	3	3	2	0.5
Co12	3	100	2	0	1	0.5
Co-64-2	4	100	5	5	2	0.5

*Values taken from Monsieurs *et al.* (2011).
†Values taken from Monchy *et al.* (2007).

environmental impact of radionuclide migration. Microbial metabolism has the potential to significantly alter the chemistry of radionuclide-contaminated environments and control radionuclide speciation and mobility, and therefore has applications in waste storage and management. It is now widely considered that the large metal uptake capacity and cheap availability of many microorganisms make them ideal candidates for industrial metal removal, and several commercial operations have adopted microorganisms-mediated systems as an important part of their detoxification process (Avery, 1995). The isolation of novel bacteria that are resistant to either Co^{2+} or Cs^{+} could prove useful in the bioremediation of nuclear fuel storage ponds.

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References

Avery SV (1995) Microbial interactions with caesium – implications for biotechnology. *J Chem Technol Biotechnol* **62**: 3–16.

Barceloux DG (1999) Cobalt. *J Toxicol Clin Toxicol* **37**: 201–206.

Barras F & Fontecave M (2011) Cobalt stress in *Escherichia coli* and *Salmonella enterica*: Molecular bases for toxicity and resistance. *Metallomics* **3**: 1130–1134.

Edgar RC (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res* **32**: 1792–1797.

Kim OS, Cho YJ, Lee K *et al.* (2012) Introducing EZTAXON-E: a prokaryotic 16S rRNA gene sequence database with phylotypes that represent uncultured species. *Int J Syst Evol Microbiol* **62**: 716–721.

Kobayashi M & Shimizu S (1999) Cobalt proteins. *Eur J Biochem* **261**: 1–9.

Kuwahara C, Fukumoto A, Nishina M, Sugiyama H, Anzai Y & Kato F (2011) Characteristics of cesium accumulation in the filamentous soil bacterium *Streptomyces* sp. K202. *J Environ Radioact* **102**: 138–144.

Lane DJ(1991). 16S/23S rRNA sequencing. *Nucleic Acid Techniques in Bacterial Systematics* (Stackebrandt E & Goodfellow M, eds), pp. 115–175. Wiley, London.

Liesegang H, Lemke K, Siddiqui RA & Schlegel HG (1993) Characterization of the inducible nickel and cobalt resistance determinant cnr from pMOL28 of *Alcaligenes eutrophus* CH34. *J Bacteriol* **175**: 767–778.

Melnikov P & Zanoni LZ (2013) Clinical effects of caesium intake. *Biol Trace Elem Res* **135**: 1–9.

Mergeay M, Monchy S, Vallaeys T, Auquier V, Benotmane A, Bertin P, Taghavi S, Dunn J, van der Lelie D & Wattiez R (2003) *Ralstonia metallidurans*, a bacterium specifically adapted to toxic metals: towards a catalogue of metal-responsive genes. *FEMS Microbiol Rev* **27**: 385–410.

Mergeay M, Nies D, Schlegel HG, Gerits J, Charles P & Van Gijsegem F (1985) *Alcaligenes eutrophus* CH34 is a facultative chemolithotroph with plasmid-bound resistance to heavy metals. *J Bacteriol* **162**: 328–334.

Monchy S, Benotmane MA, Janssen P, Vallaeys T, Taghavi S, van der Lelie D & Mergeay M (2007) Plasmids pMOL28 and pMOL30 of *Cupriavidus metallidurans* are specialized in the maximal viable response to heavy metals. *J Bacteriol* **189**: 7417–7425.

Monsieurs P, Moors H, Van Houdt R, Janssen PJ, Janssen A, Coninx I, Mergeay M & Leys N (2011) Heavy metal resistance in *Cupriavidus metallidurans* CH34 is governed by an intricate transcriptional network. *Biometals* **24**: 1133–1151.

Nies DH (1992) Resistance to cadmium, cobalt, zinc, and nickel in microbes. *Plasmid* **27**: 17–28.

Nies DH (1999) Microbial heavy-metal resistance. *Appl Microbiol Biotechnol* **51**: 730–750.

Nies DH (2003) Efflux-mediated heavy metal resistance in prokaryotes. *FEMS Microbiol Rev* **27**: 313–339.

Nies D, Mergeay M, Friedrich B & Schlegel HG (1987) Cloning of plasmid genes encoding resistance to cadmium, zinc, and cobalt in *Alcaligenes eutrophus* CH34. *J Bacteriol* **169**: 4865–4868.

Paterson-Beedle M, Macaskie LE, Lee CH, Hriljac JA, Jee KY & Kim WH (2006) Utilisation of a hydrogen uranyl phosphate-based ion exchanger supported on a biofilm for the removal of cobalt, strontium and caesium from aqueous solutions. *Hydrometallurgy* **83**: 141–145.

Ranquet C, Ollagnier-de-Choudens S, Loiseau L, Barras F & Fontecave M (2007) Cobalt stress in *Escherichia coli*. The effect on the iron-sulphur proteins. *J Biol Chem* **282**: 30442–30451.

Reasoner DJ & Geldreich EE (1985) A new medium for the enumeration and subculture of bacteria from potable water. *Appl Environ Microbiol* **49**: 1–7.

Rodrigue A, Effantin G & Mandrand-Berthelot MA (2005) Identification of *rcnA* (*yohM*), a nickel and cobalt resistance gene in *Escherichia coli*. *J Bacteriol* **187**: 2912–2916.

Saitou N & Nei M (1987) The neighbour-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**: 406–425.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M & Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* **28**: 2731–2739.

Wang Q, Garrity GM, Tiedje JM & Cole JR (2007) Naive Bayesian classifier for rapid assignment of rRNA sequences into new bacterial taxonomy. *Appl Environ Microbiol* **73**: 5261–5267.